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DETERMINATION OF N-(3-ACETAMIDOPROPYL)PYRROLIDIN-2-ONE, A METABOLITE OF SPERMIDINE, IN URINE BY ISOTOPE DILUTION MASS FRAGMENTOGRAPHY

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SUMMARY

A capillary gas chromatographic method with mass spectrometric detection for the determination of N-(3-acetamidopropyl)pyrrolidin-2-one, the monoacetyl conjugate of isoputreanine-γ-lactam, in urine has been developed. Using a quantification based on stable isotope dilution mass fragmentography, age-dependent normal values for the urinary excretion of N-(3-acetamidopropyl)pyrrolidin-2-one by 44 apparently healthy control subjects were determined. Quality control data and normal values for 27 adults are given. The method was applied to the monitoring of the chemotherapeutic treatment of two patients with high-grade non-Hodgkin lymphoma.

INTRODUCTION

Polyamines are catabolysed by the action of a multitude of enzymes. The quantitatively most important deactivating pathways are N-conjugation with acetic acid and subsequent or direct oxidation by various amino oxidases [1–4]. One of these routes includes the oxidative deamination of one or both primary amino groups, followed by intermediate aldehyde oxidation. The combined action of these enzymes gives rise to the non-α-amino acid metabolites [4,5]. After intraperitoneal administration of stable isotopically labelled spermidine to rats, labelled putreanine and isoputreanine can be demonstrated in acid-hydrolysed urines [6]. For normal humans the urinary concentration of total isoputreanine is about twice that of its precursor spermidine [7,8]. The catabolic pressure on polyamines may be considerably increased during neoplastic diseases, as even higher proportions of oxidized polyamine metabolites, especially isoputreanine, have been

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measured in urine hydrolysates of cancer patients [8,9]. Seiler and Knödgen [10] suggested that isoputreanine is predominantly excreted as the monoacetyl conjugate of N-(3-aminopropyl)pyrrolidin-2-one, the acetylated γ-lactam form of isoputreanine. This compound may be formed by oxidative deamination of N^1-acetylspermidine, the quantitatively most prominent form in which spermidine is excreted in the urine of normal healthy humans and especially cancer patients [2,10,11].

In this paper we describe the identification and quantification of N^1-acetyl-isoputreanine-γ-lactam in human urine by gas chromatography–mass spectrometry. We give quality control data and age-dependent normal values. In addition, we used the method for the follow-up of two patients with non-Hodgkin lymphoma during chemotherapy.

EXPERIMENTAL

Reagents

N-(3-Aminopropyl)pyrrolidin-2-one (the γ-lactam form of isoputreanine; Isoga) was obtained from Aldrich Europe (Beerse, Belgium), acetyl chloride from Baker (Phillipsburg, NJ, U.S.A.), trideuterated acetyl chloride from Sigma (St. Louis, MO, U.S.A.) and Carbowax 1000M from Chrompack (Middelburg, The Netherlands). All other reagents were from Merck (Darmstadt, F.R.G.).

Syntheses

N-(3-Acetamidopropyl)pyrrolidin-2-one (Acisoga) and its trideuterated analogue (Acisoga-d3) were synthesized by dissolving the free base of Isoga in methanol and subsequent acetylation with one molar equivalent each of acetyl chloride and trideuterated acetyl chloride. After the addition of an equivalent volume of 0.5 mol/l phosphate buffer (pH 7.0), the pH of the mixture was adjusted to 7.0 with 4 mol/l sodium hydroxide solution. The reaction products were extracted into dichloromethane and the dichloromethane layer was dried with anhydrous sodium sulphate. The solutions were stored at 4°C. The purity was checked with gas chromatography–mass spectrometry. After hydrolysis of part of the reaction product in 6 mol/l hydrochloric acid at 120°C for 18 h, quantification was performed on the basis of the heptafluorobutyryl derivative of Isoga by gas chromatography with nitrogen–phosphorus detection [12].

Samples

Urine samples from healthy persons and patients with non-Hodgkin lymphoma during chemotherapeutic treatment were collected either in polyethylene bottles (24-h urines) or without delay acidified to pH 1–2 (untimed voidings). During collection of 24-h urines the samples were kept at 4°C. Aliquots were stored at −20°C until taken for analysis.

Pre-purification. A 1-ml volume of urine was added to a tube containing 10 nmol of Acisoga-d3 (200 μl of a solution containing 0.05 mmol of Acisoga-d3 per litre of dichloromethane, evaporated to dryness at room temperature under a stream of air). Subsequently, 1 ml of 0.5 mol/l phosphate buffer (pH 7.0) was

added. The contents of the tube were carefully mixed in an ultrasonic bath for 10 min. Acisoga and Acisoga-d3 were extracted into 5 ml of dichloromethane. The dichloromethane (lower) layer was dried by treatment with a small amount of anhydrous sodium sulphate, and subsequently evaporated to dryness at room temperature under a stream of air. The residue was dissolved in 100 μl of dichloromethane containing 2 g/l of Carbowax 1000M. A 2-μl aliquot was analysed by gas chromatography with mass spectrometric detection.

Equipment. Gas chromatography–mass spectrometry was performed with a Varian 3700 gas chromatograph directly coupled to a MAT 44-S mass spectrometer. The combination was operated in the ammonia chemical ionization mode, under the following conditions: helium flow-rate, 1.0 ml/min; injector temperature, 250°C; splitting ratio, 1:10, oven temperature programme, 200°C, 10°C/min to 260°C; ion source temperature, 200°C; and ionization energy, 180 eV. The column was a 25 m$\times$0.32 mm I.D. CP-Sil-19 coated (0.2 μm film thickness) fused-silica capillary (Chrompack).

Quantification and quality control. We monitored the ions at *m/z* 185 and 188, corresponding to the $[M+H]^+$ ions of naturally occurring Acisoga and its trideuterated analogue, respectively. The peak-area ratio of the ions at *m/z* 185 and 188 was calculated using a Finnigan-MAT SS-200 data system. Concentrations were computed by means of a calculated calibration graph, composed of the data from the corresponding peak-area ratios of various amounts of Acisoga (range 0–25 nmol) added to a fixed amount (10 nmol) of its deuterated analogue. Concentrations were expressed relative to that of urinary creatinine. Creatinine was measured by a picric acid method [13]. For quality control, we analysed in each series a 1-ml aliquot of a pooled urine, and the same pooled urine enriched with 10 nmol of Acisoga.

RESULTS AND DISCUSSION

Method and quality control

Fig. 1 shows mass fragmentograms of Acisoga and its trideuterated analogue monitored at *m/z* 185 and 188, respectively, for a standard (S), and urine samples of a normal healthy adult (N) and a patient with non-Hodgkin lymphoma (P). Ammonia chemical ionization mass spectrometry of Acisoga and its deuterated analogue results almost exclusively in the formation of the $[M+H]^+$ ion. The detection limit is estimated to be about 50 pmol of injected Acisoga with a signal-to-noise ratio of 6.

The within-series precision and recovery for the determination of Acisoga were investigated by analysing six aliquots of pooled urine from normal adults, together with the same sample enriched with 10 μmol of Acisoga per litre. The mean concentration was 3.84 μmol/l [coefficient of variation (C.V.), 2.3%; range, 3.70–3.98 μmol/l). The mean recovery was 99.4% (C.V., 4.0%; range, 95.1–105.2%).

The day-to-day precision and recovery of the method were calculated from the same pooled urine determined in six series that were analysed during a period of 4 months. The results were a mean endogeneous concentration of 3.77 μmol/l

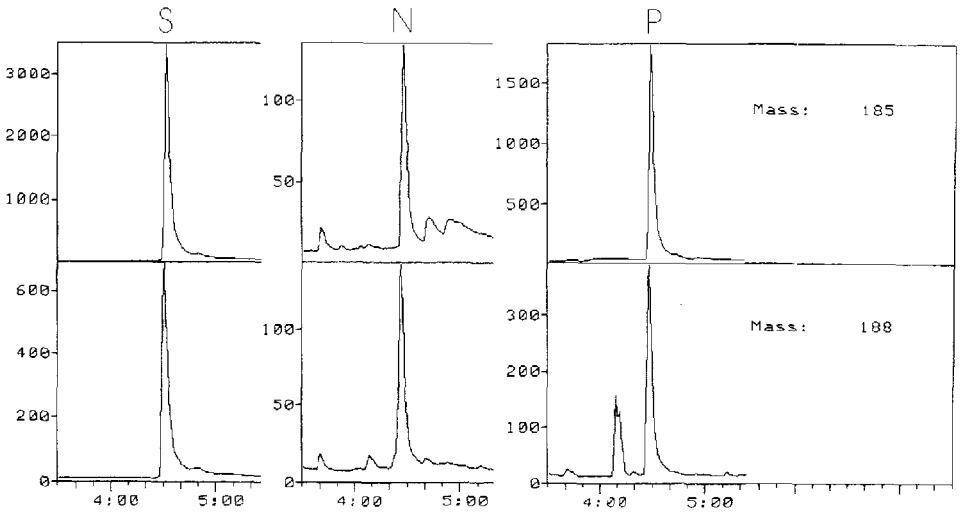

Fig. 1. Mass fragmentograms of N-(3-acetamidopropyl)pyrrolidin-2-one (Acisoga; m/z 185) and its trideuterated internal standard (m/z 188) in a standard (S), and urine samples of a healthy adult (N) and a patient with high-grade non-Hodgkin lymphoma (P). Time scale in minutes and peak intensity in arbitrary units (counts).

(C.V., 3.2%; range, 3.55–3.91 μmol/l) and a mean recovery of 97.5% (C.V., 5.6%; range, 89.5–105.5%).

Normal values

Fig. 2 shows age-dependent normal values for the urinary excretion of Acisoga and the sum of isoputreanine (Isoputr) and Isoga expressed in terms of creatinine, together with the derived acetylation percentage (Acisoga as a percentage of the sum of Isoputr, Isoga and Acisoga). The values for the sum of Isoputr and Isoga were determined by a previously described gas chromatographic method with nitrogen–phosphorus detection [12]. We only considered the sum of Isoputr and Isoga, as with that method it was impossible to establish whether Isoga is a normal urinary excretory product or whether it is formed as a derivatization artefact [12]. A gradually decreasing excretion with age was established for Acisoga, which is merely caused by the rapid increase in creatinine excretion during the first years of life, and should therefore be considered as "false" [14]. On the other hand, with increasing age a steep decrease in the excretion of the sum of Isoputr and Isoga was found, which may only partly be attributed to the mentioned increase in creatinine. Consequently, the acetylation percentage (see Fig. 2) increased with age, which may be due to age-dependent shifts in the polyamine-degrading routes. An increase in the activities of polyamine-acetylating enzymes with age seems unlikely, as no age dependence in urine was found for the acetylation percentage of spermidine (i.e., acetylated spermidine as a percentage of the sum of free and acetylated spermidine) [12].

Table I summarizes the urinary excretion levels for a normal adult population (ages 15–77 years), together with the acetylation percentage. A comparison between the sum of normal urinary concentrations of Isoputr, Isoga and Acisoga

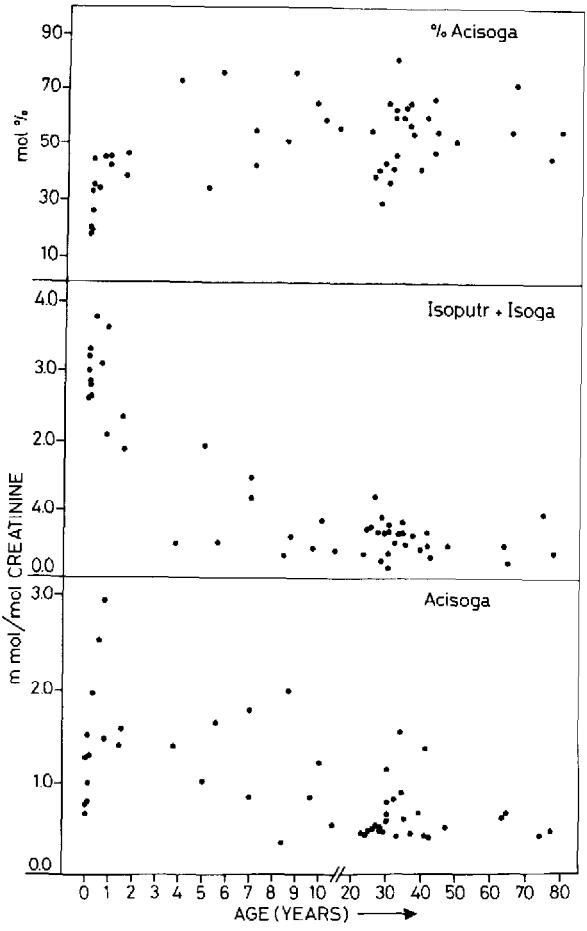

Fig. 2. Values for normal excretion of Acisoga, the sum of Isoga and Isoputr (in mmol/mol of creatinine) and the percentage of acetylation (in mol-%) for 44 healthy persons (ages 4 days to 77 years). mol-% (percentage of acetylation), the amount of Acisoga as a percentage of the sum of Acisoga, Isoga and Isoputr.

TABLE I

CONCENTRATIONS OF ISOPUTREANINE AND ITS DERIVATIVES (mmol/mol CREATININE) IN THE URINE OF 27 APPARENTLY HEALTHY ADULTS (AGES 15–77 YEARS)

Compound	Mean	Range	C.V. (%)
Acisoga	0.65	0.41–1.57	44.9
Isoputr + Isoga★	0.64	0.18–1.20	36.1
Acisoga + Isoputr + Isoga	1.25	0.74–2.41	32.0
Total Isoputr★★	1.31	0.64–2.77	32.9
% Acisoga★★★	53.5	29–90	22.2

★Ref. 12.

★★In acid-hydrolysed urines [7], ages 13–72 years, $n = 52$.

★★★Acisoga as a percentage of the sum of Acisoga, Isoputr and Isoga.

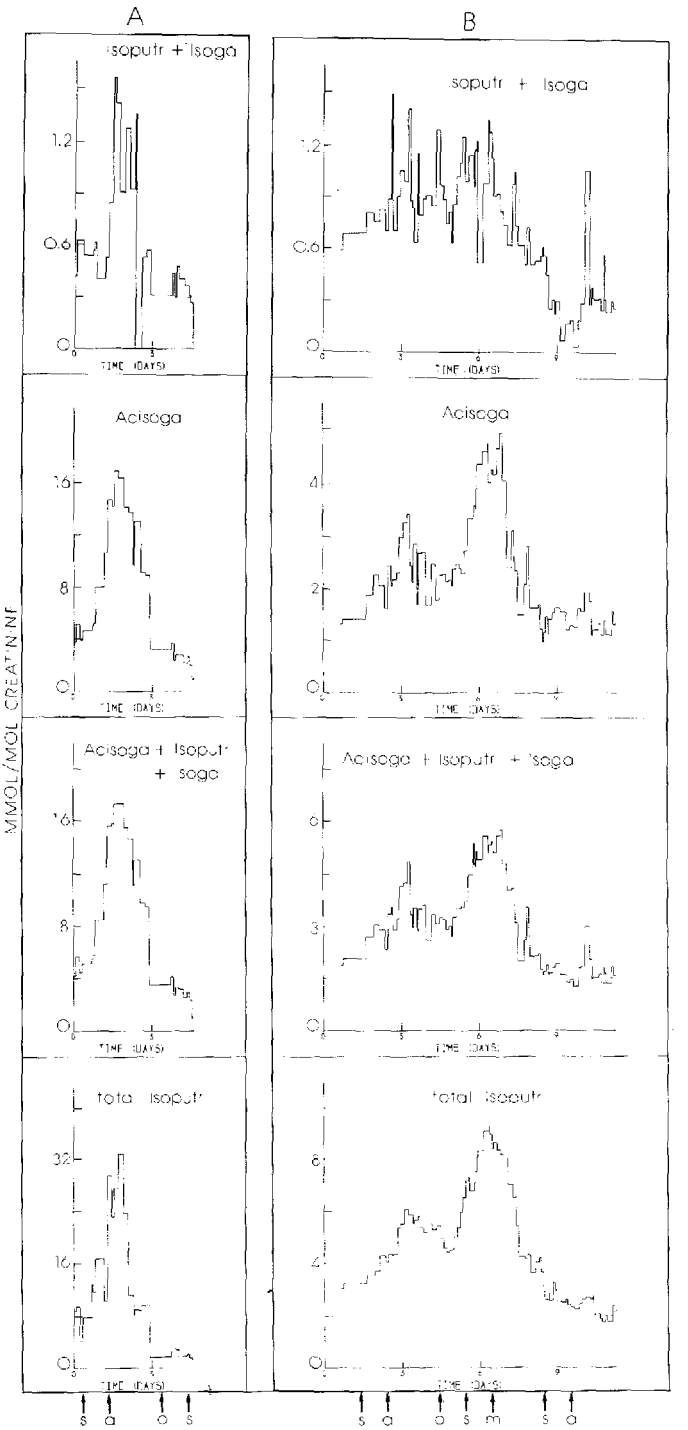

Fig. 3. Follow-up curves of Acisoga, the sum of Isoga and Isoputr, the sum of Acisoga, Isoga and Isoputr in unhydrolysed urines and of total Isoputr in acid-hydrolysed urines of two patients with stage IV high-grade non-Hodgkin lymphoma during treatment with a new chemotherapeutic scheme. Measurements were performed on every spontaneously voided urine sample. Data are expressed in mmol/mol of creatinine as a function of time (days) . (A) 16-year old girl; (B) 63-year-old man. s, Solumedrol (methylprednisolone); a, cytosine arabinoside; o, oncovin (vincristine); m, methotrexate. Note the difference in the scales of the ordinates.

and the previously reported normal concentration of Isoputr in acid hydrolysed urines [7] is made. The concentration of the free plus acetylated forms is in reasonable agreement with the total Isoputr concentration, suggesting that in the normal situation Isoputr is excreted as Acisoga and Isoputr and probably Isoga (see above). The latter has previously been identified by Seiler and Knödgen [10].

Clinical applications

The quantitatively and qualitatively most important polyamines in normal and tumour cells are spermidine and spermine [15]. Spontaneous or therapeutically induced cell death leads to the liberation of polyamines. Subsequently they may be (1) excreted into the urine directly (a quantitatively minor route [12]), (2) metabolized (conjugated, catabolized or both) and excreted into the urine or (3) taken up for re-use (salvage pathway). High concentrations of circulating polyamines are toxic. There are strong indications [6,8] that an increased polyamine liberation leads to the induction of polyamine-catabolizing enzymes. The quantitatively most important catabolite in acid-hydrolysed urines is Isoputr [7], originating from spermidine. In the normal situation Isoputr is mostly excreted as Acisoga (see Table I).

Concentrations of Acisoga were determined in urine samples of two patients with stage IV high-grade non-Hodgkin lymphoma during successful treatment with a new chemotherapeutic remission induction scheme. Both patients were clinically classified as complete responders to therapy. To obtain detailed information, we made measurements in every spontaneous voiding (constant monitoring concept). Fig. 2 shows the results, together with data for the sum of Isoga and Isoputr [12], the sum of Isoga, Isoputr and Acisoga, and total Isoputr after acid hydrolysis [7]. In contrast to the normal situation (see Table I), the sum of Isoputr and Isoga amounted to only a minor part (about 15%) of the total Isoputr in acid-hydrolysed urine. During therapy the kinetics of Acisoga and total Isoputr were similar. However, in the period of increased excretion of total Isoputr (Fig. 3; see also Table I) the sum of the concentations of Acisoga, Isoga and Isoputr for patient A amounted to only 50% of the concentration of total Isoputr, and to about 65% for patient B. With the normalization of total Isoputr levels the discrepancy between the sum of Isoputr, Isoga and Acisoga, and total Isoputr disappeared. These data suggest that during enhanced levels of total Isoputr other forms of conjugated Isoputr than Acisoga and Isoga are also excreted.

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